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Molecular taxonomy of the alpine bullhead *Alpinocottus poecilopus* (Heckel, 1837) from the Pomeranian enclave

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Original article

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The alpine bullhead (*Alpinocottus poecilopus*) is a cold-water benthic fish with a disjunct distribution across Europe, including a recently discovered population in northern Poland's Pomeranian region. Prior to now, the taxonomic identity of this enclave population has not been confirmed using molecular methods. This study presents the first DNA barcoding analysis of the *A. poecilopus* from Pomerania. Sequences of the mitochondrial *coI* gene and phylogenetic reconstruction confirmed the species identity and revealed no, or a very low genetic divergence between the Pomeranian and the Scandinavian and Czech Republic *A. poecilopus*. The results also support the recent reclassification of *A. poecilopus* into the genus *Alpinocottus*, alongside *A. volki* and *A. szanaga*, forming a distinct clade with *C. kolymensis*, which may also belong to this genus. These findings confirm that the Pomeranian alpine bullhead represents a genetically valid relict population, which was likely preserved due to postglacial isolation and specific cold-water habitat conditions. Additionally, unverified reports of an alpine bullhead occurrence in the Drwęca River basin (Vistula system) were clarified, as specimens from this site were identified as the European bullhead (*Cottus gobio*).

Key words: bullheads, barcode analysis, phylogenetic, Cottidae.

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The formerly known *Cottus poecilopus* (Heckel, 1837) has recently been allocated to a new genus *Alpinocottus*, together with other two species: *Alpinocottus volki* (Taranetz, 1933) and *Alpinocottus szanaga* (Dybowski, 1869) (Bogdanov 2023; Froese & Pauly 2025).

In Europe, the alpine bullhead *Alpinocottus* species occur in two main regions. The first is the Carpathian-Sudetic area, where it inhabits mountain streams in the upper Vistula and Odra river basins, as well as the Carpathian parts of the Dniester and Danube systems. The second area is the Scandinavian region, where its geographic range is divided into a northern part covering Finland, northeastern Sweden and parts of northern Norway, and a southern part that includes southern Sweden, southwestern Norway and the Jutland Peninsula (Andreasson 1972; Koli 1957; Nybelin 1969; Pethon 1967; Witkowski 2005). Apart from these two regions, several isolated lakes where the alpine bullhead previously occurred are known in Central and Eastern Europe: Ładoga and Onega in Russia; Hańcza and Ińsko in Poland; and Luzin, Schwerin and Grosser Plöner in Germany (Bauch 1970; Berg 1949; Duncker 1925; Witkowski 1975). Currently, from the German localities, a residual population exists only in Lake Luzin and is undergoing a restitution programme (Kotusz *et al.* 2004; Krappe *et al.* 2021). In Poland, a relatively large population exists in Lake Hańcza (Kotusz *et al.* 2004).

Recently, in 2005, new localities of the alpine bullhead were recorded in an enclave in the Pomerania region (northern Poland) from three catchments of the Reda, Łeba and Łupawa Rivers (Radtke *et al.* 2005). It is considered to be an enclave because detailed fish faunistic studies conducted in all neighbouring rivers of the Pomeranian catchments have revealed the presence of only the European bullhead *Cottus gobio* (Radtke *et al.* 2017). Currently, the phylogenetic position of the alpine bullhead populations from northern Polish rivers has not been assessed, and no genetic analysis was conducted prior to the current date. As a result, the species was assigned based only on morphometric characteristics (Radtke *et al.* 2005; Krappe *et al.* 2021).

The main aim of this study is to determine the generic position of the alpine bullhead from the Pomeranian region based on a barcoding analysis of the cytochrome oxidase subunit I (*coI*).

Material and Methods

Ethical approval

All procedures complied with the Polish regulations and ethical standards. Fieldwork was approved by the Departments of Environmental Protection of the Pomeranian and Kuyavian-Pomeranian Voivodeships. No additional ethical clearance was required.

Study area and sampling

The fish collected for the analysis were caught during autumn electrofishing (backpack device, 115-250 V, 1-2 A, DC) from six localities. The captured fish were anesthetised with etomidate, and small fin clip (approximately 2-5 mm^2) was collected from every specimen for the molecular analysis and stored in 95% ethanol. The samples taken were described and are stored in the laboratory of the Department of Migratory Fish in Rutki, NIFRI. After awakening, all fish were released at the site of capture. Five of the electrofished sites were situated in adjacent coastal river basins (Reda, Łeba and Łupawa), where the alpine bullhead was recently discovered (Fig. 1, Table 1). One site was situated in

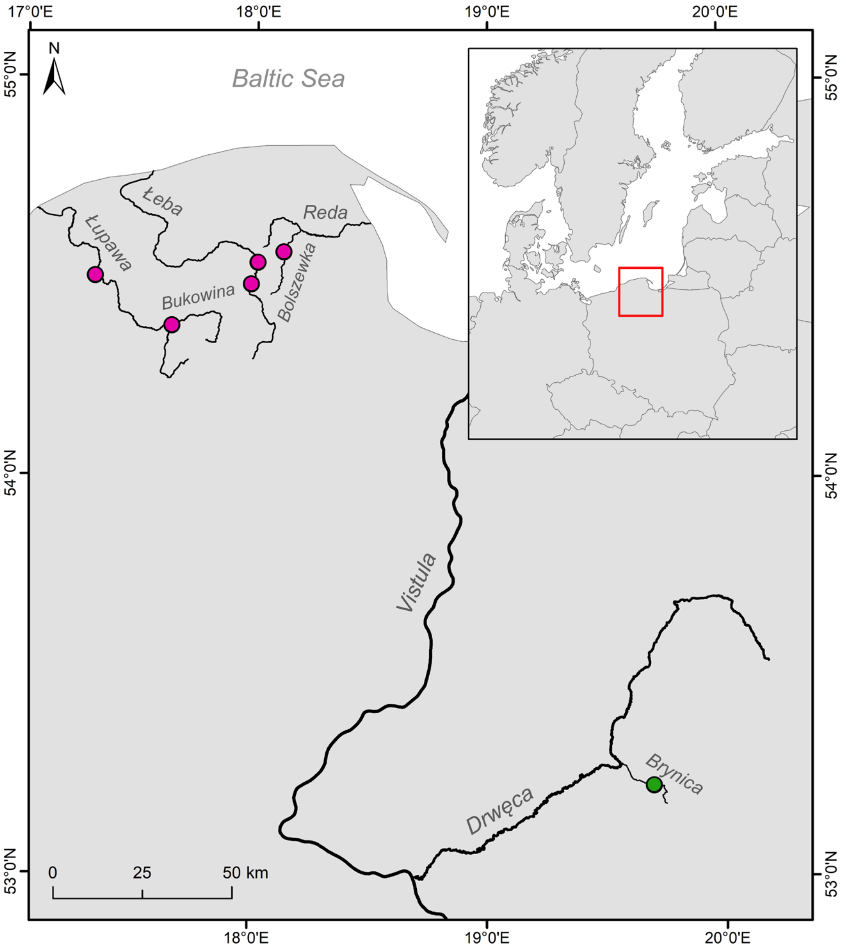

Fig. 1. Sampling locations of the *A. poecilopus* (pink) and *C. gobio* (green).

Table 1

Site description of the samples from this study. From left: river/stream name, N = number of analysed specimens, date of the collected sample (D/M/Y), locality description, coordinates and species name

River	N	Date	Locality	GPS	Species
Bolszewka	5	21.10.2020	Luzino	54.565002, 18.122592	*A. poecilopus*
Łeba_1	3	21.10.2020	Osiek	54.487456, 17.985775	*A. poecilopus*
Łeba_2	5	21.10.2020	Paraszyno	54.536638, 18.012001	*A. poecilopus*
Bukowina	5	11.10.2023	Kozin	54.381538, 17.642362	*A. poecilopus*
Łupawa	3	05.10.2020	Damnica	54.502853, 17.310189	*A. poecilopus*
Brynica	5	14.10.2022	Traczyska	53.231239, 19.701867	*C. gobio*

the Drwęca River basin (a tributary of the lower Vistula), where previous reports of *A. poecilopus* had been uncertain (Backiel 1964; Witkowski 1996).

The studied coastal rivers originate in the Kashubian Lakeland, at elevations exceeding 200 metres above sea level. The areas of the river catchments are as follows: Reda – 1546 km²; Łeba – 1768 km²; and Łupawa – 924 km². In the Reda catchment, sampling was conducted at one site in its largest tributary, the Bolszewka River. In the Łeba catchment, samples were taken from two localities in its middle portion, in the area of Osiek and Paraszyno Villages. In the Łupawa system, samples were collected from its middle portion, as well as from its largest right-bank tributary, the Bukowina stream. In the Drwęca River catchment, samples were collected from the Brynica stream, a small left-bank tributary (Fig. 1, Table 1). The river localities varied in their ecomorphology: river widths ranged from 3.7 to 15.5 metres, while the average depths ranged from 0.2 to 0.6 metres. The surveyed sections were characterised by steep gradients (>2‰), numerous rapids and by substrates dominated by gravel and stones.

Molecular analysis of the *coI* gene

An analysis of subunit I of the cytochrome oxidase (*coI*) barcode sequences from 21 specimens of the alpine bullhead genus *Alpinocottus* from five localities was used to genetically assign the species identification and investigate the phylogenetic position (Table 1). Additionally, five individuals of the European bullhead of the *Cottus gobio* group from the Drwęca River (Vistula catchment) were included as an outgroup.

The DNA extractions were performed with Genomic Mini Kits (A&A Biotechnology, Poland). The purity and concentration of the DNA eluates were assessed on 1.5% agarose gel and with a NanoDrop spectrophotometer (Thermo Fisher Scientific). The primers used to amplify the *coI* gene were the following: COI-Fish-F 5'-TTCTCAACTAACCAYAAAGAYATYGG-3', COI-Fish-R 5'-TAGACTTCTGGGTGGCCRAARAAYCA-3' (Ward *et al.* 2005; Kochzius *et al.* 2010). The *coI* polymerase chain reaction (PCR) with a total volume of 20 µl contained 10x reaction buffer, 1 mM dNTPs, 2.5 mM $MgCl_2$, 0.33 µM of each primer, 1.25 U Tag Polymerase (Run A&A Biotechnology, Poland) and 3 µl of DNA-extract. The PCR temperature protocol followed Kochzius *et al.* (2010). The results of each PCR were confirmed by separating the analysed samples in 1.5% agarose gel, and each PCR product was then sequenced bidirectionally according to Sanger's method (Genomed, Poland). The sequences obtained were analysed with MEGA 11 (Tamura *et al.* 2021) and were deposited in the GenBank National Centre for Biotechnology Information (NCBI) under the following numbers: PV335166 to PV335186 (*Alpinocottus poecilopus* from the Bolszewka, Łeba, Bukowina and Łupawa Rivers), and PV339446 to PV339450 (*Cottus gobio*, from the Brynica River) (Supplementary Material SM.01.). Consensus sequences were then generated from all obtained sequences for both species in the BioEdit 7.0.5.3 software (Hall 1999). Species matching was performed using the BLAST tool, assuming the highest value of taxonomic percent identity and query cover.

Identification, phylogenetic analysis and divergence

To access the phylogenetic position of the specimens of *Alpinocottus* generated in this study, 634 bp of new *coI* sequences were concatenated with 25 Cottidae species downloaded from GenBank from previous studies (Ao *et al.* 2016; Balakirev *et al.* 2016; Dziedzic *et al.* 2023; Ericson *et al.* 2020;

Mugue *et al.* 2021; Radchenko *et al.* 2023; Sandel *et al.* 2017; Sheraliev & Peng 2021; Teterina *et al.* 2021; Verissimo *et al.* 2025; Zangl *et al.* 2022). The alignment was performed with MAFFT v6.861b with the default options (Katoh *et al.* 2005). The phylogenetic analysis was performed using the 'build' function of ETE3 3.1.3 (Huerta-Cepas *et al.* 2016), as implemented on the GenomeNet (https://www.genome.jp/tools/ete/). The Maximum Likelihood (ML) tree was inferred using IQ-TREE 1.5.5 through ModelFinder and a tree reconstruction (Nguyen *et al.* 2015). The best-fit model for the *coI* dataset according to BIC was Blosum62+F+R3. The tree branches were accessed by SH-like aLRT with 1000 replicates.

Additionally, estimates of the evolutionary divergence between the *Alpinocottus* sequences were calculated using the Maximum Composite Likelihood model (Tamura *et al.* 2004) with codon positions of 1st+2nd+3rd+Noncoding. Ambiguous positions were removed for each sequence pair (pairwise deletion option). Evolutionary analyses were conducted in MEGA11 (Tamura *et al.* 2021).

Results

The taxonomic identification based on the *coI* gene fully confirmed all the previous specimen assignments. In both *A. poecilopus* and *C. gobio*, the sequences obtained showed a 99.5% to 100% probability of species identity (Table 2). The second-highest genetically closest species of *A. poecilopus* was *Cottus kolymensis* (96.5% similar) (Sideleva and A. Goto, 2012), and for *C. gobio* was *C. microstomus* (100% similar) (Heckel, 1837) (see Table 2).

The *Alpinocottus* sequences from the Pomeranian Rivers showed no *coI* divergence (Table 3), as well as compared to the Sweden and the Czech Republic populations (Table 3). The interspecific variation of *coI* of *Alpinocottus* species ranged from 0.03 to

Table 2

Details of the cytochrome oxidase subunit 1 (*coI*) sequences obtained: bullhead species, sequence length (bp = base pairs), probability of species identification (taxon identity in %), GenBank accession numbers and best next species similarity (congener similarity)

Species	bp	Taxon identity	Accession numbers	Congener similarity
Alpinocottus poecilopus	652	99.5%	PV335166 to PV335186	*Cottus kolymensis* (96.5%)
Cottus gobio	652	100%	PV339446 to PV339450	*Cottus microstomus* (100%)

Table 3

Estimates of evolutionary pairwise distances (divergence) between sequences from the genus *Alpinocottus* including *C. kolymensis*. Numbers show the base substitutions per site between sequences

Localities/ species	Bukowina River	Łeba River1	Łeba River2	Łupawa River	Sweden *A. poecilopus*	Czech Republic *A. poecilopus*	Russia *C. kolymensis*	Russia *A. szanaga*	Russia *A. volki*
Bolszewka River	0.0048	0.0032	0.0032	0.0048	0.0034	0.0034	0.0315	0.0581	0.0741
Bukowina River		0.0034	0.0034	0.0017	0.0034	0.0034	0.0337	0.061	0.0691
Łeba River1			0	0.0017	0	0	0.0314	0.0581	0.0701
Łeba River2				0.0017	0	0	0.0314	0.0581	0.0701
Łupawa River					0.0017	0.0017	0.0332	0.06	0.072
Sweden *A. poecilopus*						0	0.0314	0.0581	0.0701
Czech Republic *A. poecilopus*							0.0314	0.0581	0.0701
Russia *C. kolymensis*								0.0504	0.0681
Russia *A. szanaga*									0 .0904

0.07, showing that *C. kolymensis* is more similar to *A. poecilopus* (0.03).

The phylogenetic analysis revealed two major groups. The first group consists of the alpine bullhead Polish populations of *Alpinocottus* species from the Pomeranian rivers and the populations from Sweden and Czech Republic, and the Kolyma bullhead *Cottus kolymensis* (Fig. 2), a sister clade to other two species *A. szanaga* and *A. volki* from Russia. This group is sister to a second larger clade formed by three subgroups: the first group consists of the *Cottus* species distributed in the North America and the Aleutian Islands (*C. aleuticus*, *C. pitensis*, *C. perplexus*, *C. asper* and *C. gulosus*); the second group is comprised of *Cottus* bullheads from Canada, Uzbekistan, eastern China and part of Europe, including species from Poland, Austria, Germany and Spain (*C. ricei*, *C. spinulosus*, *C. duzungaricus*, *C. gobio*, *C. hispaniolensis* and *C. rheneanus*, respectively); and the third group consists of *Cottus* species (*C. confuses* and *C. bairdii*) from the mid to northern United States, which is a sister clade of the bullhead species from central Russia and Baikal (*P. major, C. grewingki, C. inermis, C. baicalensis, B. nikolskii, B. multiradiatus* and *B. talieri*) (Fig. 2).

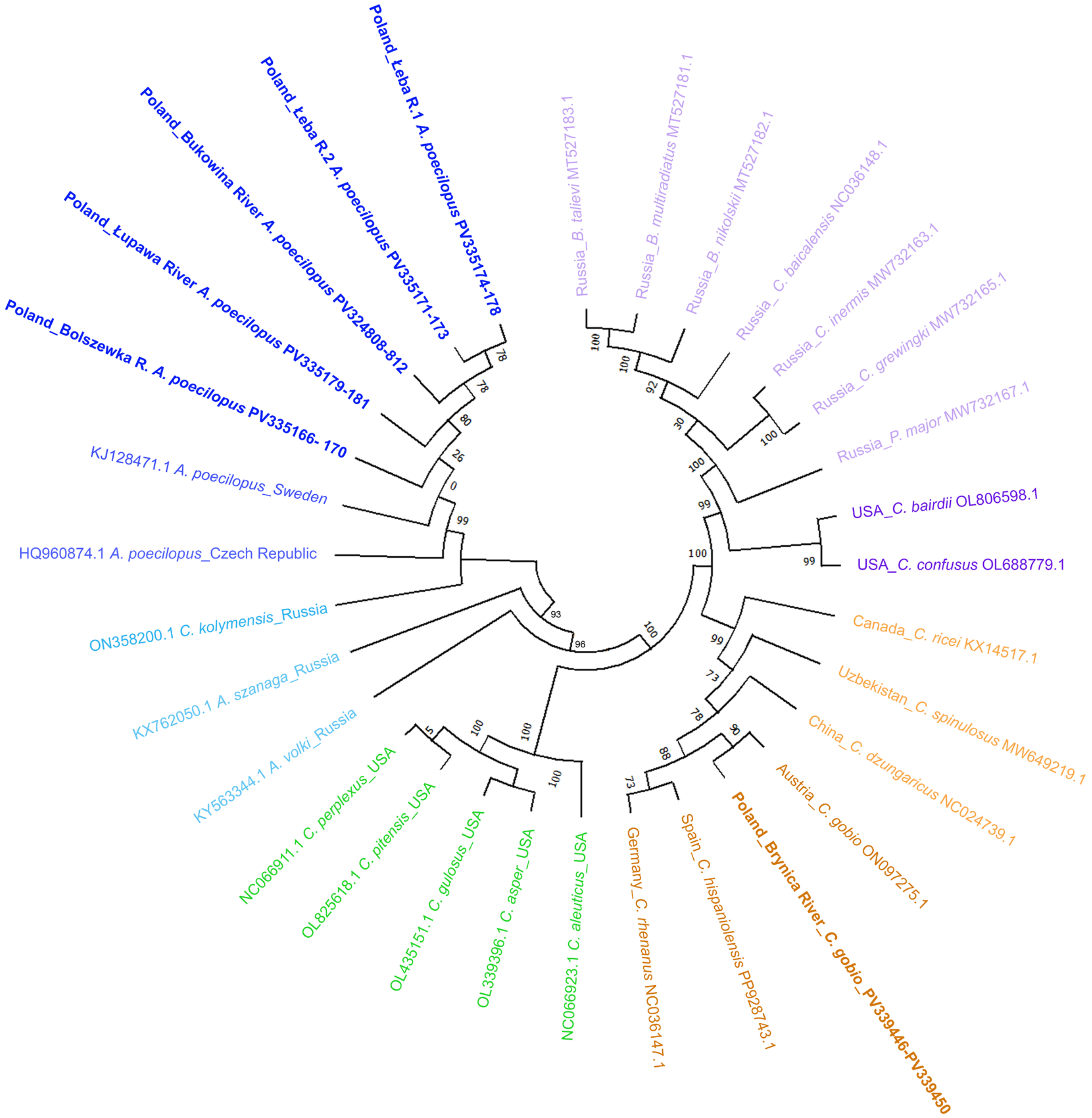

Fig. 2. Maximum Likelihood phylogenetic tree of barcoding sequences of five Polish populations of the alpine bullhead and the Drwęca River population of the European bullhead, as well as 25 sequences of the bullhead (Cottidae) collected in GenBank. The values shown in the clade node represent bootstrap values.

Discussion

The results obtained from the analysis of the barcoding sequences unequivocally genetically assign the specimens previously identified morphologically in this study as *Alpinocottus poecilopus*, with the sequences available in GenBank. This resolves the prior uncertainty regarding the species' presence in the Pomeranian enclave (Krappe *et al.* 2021). Notably, the current distribution of these species in northern Polish rivers is very narrow and is limited only to parts of the catchments of three rivers: the Reda, Łeba and Łupawa. This raises a question regarding why the species has persisted in the fish fauna of this particular region. One explanation lies in the postglacial history of the area, especially the presence of the Reda-Łeba Ice-Marginal Valley, as one of the most prominent glacial meltwater valleys in the South Baltic Coast region, through which waters flowed during the retreat of the Baltic glaciation in present-day Poland (Augustowski 1965; Uścinowicz 1999). Additionally, the species' persistence may be attributed to the uniquely low thermal regime of waters in this area, which is critical for the survival of *A. poecilopus* (Radtke & Bernaś 2025).

The phylogenetic analysis of the obtained sequences of *A. poecilopus* and those deposited in GenBank revealed it to be a sister taxa of populations from Sweden and Czech Republic. The latter group is sister to *Cottus kolymensis,* and this large clade is most closely related to the *Alpinocottus szanaga* and *Alpinocottus volki* from Russia. The phylogenetic relationships between these species could indicate that *C. kolymensis* represents a species of the genus *Alpinocottus*; however, a detailed study should be conducted to investigate the generic assignment of this species. This would require a further analysis based on larger genetic sampling, as well as detailed morphological comparisons. Additionally, the validity of *Alpinocottus* as a distinct genus (Bogdanov 2023) is herein supported based on the *coI* gene. This result also does not contradict the results of the phylogenetic relationships based on the control region sequences (Shedko & Miroshnichenko 2007; Yokoyama *et al.* 2008).

The analysis of bullhead sequences of *Cottus gobio* from the Drwęca River basin (PV339446 to PV339450) was herein examined clustered with *Cottus gobio* populations from Austria, thereby confirming its identity. It is also noteworthy that the *coI* sequences of *C. gobio* and *Cottus microstomus* are almost identical, as there were virtually no differences between the two species in the studied fragment of mitochondrial DNA. This may suggest a close relationship between the two species, both of which belong to the European *Cottus gobio* complex (Freyhof *et al.* 2005; Sideleva *et al.* 2022).

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Author Contributions

Research concept and design: R.B., G.R.; Collection and/or assembly of data: R.B., G.R., T.H., A.W.-B., W.Ś.; Data analysis and interpretation: R.B., G.R., T.H., A.W.-B.; Writing the article: R.B., G.R.; Critical revision of the article: R.B.; Final approval of article: R.B.

Conflict of Interest

The authors declare no conflict of interest.

Supplementary Materials

Supplementary Materials to this article can be found online at:

http://www.isez.pan.krakow.pl/en/folia-biologica.html

Supplementary files:

SM.01. Table S1. A detailed list of the sampled *A. poecilopus* and *C. gobio* stored in the laboratory of the Department of Migratory Fish, National Inland Fisheries Reaserch Institute, Poland and whose coI gene sequences are deposited in GeneBank.

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